J. Mol. Biol. (1970) **47**, 317–333

Induction of Virus Multiplication in 3T3 Cells transformed by a Thermosensitive Mutant of Polyoma Virus

II. Formation of Oligomeric Polyoma DNA Molecules

FRANÇOIS CUZIN† MARGUERITE VOGT, MARIANNE DIECKMANN‡ AND PAUL BERG‡

The Salk Institute for Biological Studies
Post Office Box 1809, San Diego, Calif. 92112, U.S.A.

(*Received 27 May 1969, and in revised form 6 October 1969*)

3T3 cells transformed with a thermosensitive mutant of polyoma (*Ts-a*) can be induced to produce virus if the cells (Ts-a-3T3) are transferred from 38·5°C to 31°C (Vogt, 1970). Viral DNA synthesis is detectable in the activated cell population 24 hours after the shift to low temperature and steadily increases for several days. Following activation at 31°C, cultures continue to make viral DNA for at least 24 hours after they are shifted back to high temperature. Our data suggest that induction of viral multiplication involves the asynchronous occurrence of a unique event, after which DNA synthesis can continue even under non-permissive conditions.

Superhelical closed circular dimers and trimers of polyoma DNA are synthesized as well as monomeric molecules. These oligomers amount to 40% of the total viral DNA; they are not obligatory precursors of the monomeric form, which is the only type found in mature virions. Oligomers are not observed (1% or less) after infection of 3T3 cells with either wild type or the *Ts-a* mutant of polyoma. Several possible models to explain the origin of oligomers, the state of viral DNA in transformed cells and the nature of the activating event are discussed.

1. Introduction

In the accompanying paper, Vogt (1970) has described an unusual type of polyoma-transformed mouse cell. This clone, Ts-a-3T3, was recovered following infection of the normally permissive 3T3 cells (Todaro & Green, 1963) with Fried's (1965*a*) thermosensitive mutant of polyoma (*Ts-a*) at high temperature. Ts-a-3T3 can be propagated, and maintains the transformed phenotype, at 38·5°C, but if the culture is shifted to 31°C virus multiplication is induced in a significant fraction of the cells. In this paper we analyze the intracellular events, particularly the physical state and replication of the viral DNA, following the shift of a culture of Ts-a-3T3 from 38·5 to 31°C.

2. Methods

The origin of the cell lines and virus stocks, as well as the culture conditions used throughout this study, are described in the preceding paper (Vogt, 1970).

(a) *Purification of virus*

Polyoma virus was isolated from approximately 10^8 producing cells by freezing and thawing (3 times) the pooled cell debris and culture fluid. Cell debris was removed by

† Present address: Service de Génétique Cellulaire, Institut Pasteur, Paris, France.

‡ Present address: Department of Biochemistry, Stanford University School of Medicine, Stanford, Calif. 94305, U.S.A.

centrifugation at 10,000 *g* for 15 min. Virus was collected from the supernatant fluid by centrifugation at 70,000 *g* for 2·5 hr in a Spinco rotor no. 30; after resuspension in 3 ml. of 0·01 M-Tris buffer, pH 7·4, containing 0·005 M-$MgCl_2$, the mixture was incubated for 15 min at 37°C in the presence of pancreatic DNase (1 μg/ml.), RNase (1 μg/ml.) and sodium deoxycholate (0·1%). DNase action was stopped by addition of EDTA (final concentration of 0·01 M) and the virus suspension was layered onto 26 ml. of a 5 to 20% sucrose gradient containing 0·01 M-Tris buffer (pH 7·4), 0·01 M-EDTA, 1 M-NaCl, and centrifuged in the SW25-1 rotor at 25,000 rev./min for 90 min. 1-ml. fractions were collected from the bottom of the tube, and those containing the virus band were pooled and dialyzed against 0·01 M-Tris buffer, pH 7·4, containing EDTA (0·001 M). For analytical runs, 4·6-ml. sucrose gradients were centrifuged in the SW39L rotor (30,000 rev./min, 40 min).

Isolation of DNA from the virus particles was done by three successive extractions with phenol saturated with 0·25 vol. of 0·5 M-Tris buffer, pH 7·4; the aqueous layer was extracted once with chloroform and then dialyzed against several changes of Tris–EDTA buffer, pH 8·0 (0·01 M-Tris–0·005 M-EDTA).

(b) *Labeling and analysis of viral DNA*

To Petri plate cultures of cells (in modified Eagle's medium, supplemented with 10% dialyzed calf serum), [^{3}H]thymidine (10 c/m-mole, 500 μc/ml.) was added to a final concentration of 10 μc/ml. Selective extraction of viral DNA from infected or activated Ts-a-3T3 cells was accomplished according to Hirt (1967): in this procedure chromosomal DNA is precipitated with sodium dodecyl sulfate in 1 M-NaCl while the superhelical polyoma DNA remains in the supernatant solution. Reconstruction experiments using [^{3}H]thymidine-labeled cells (uninfected or non-activated) mixed with ^{32}P-labeled polyoma virus or DNA showed that this procedure removes more than 98% of the cell DNA and permits recovery of better than 85% of the viral DNA. The amount of viral DNA and the distribution of oligomeric forms was determined by velocity sedimentation in either neutral CsCl gradients or alkaline CsCl gradients according to the procedure of Dulbecco & Vogt (1963); in some cases, the extracted material was centrifuged to equilibrium in a CsCl gradient containing ethidium bromide. The details for each of these procedures are as follows:

(i) *Neutral CsCl gradient*

A sample (0·1 to 0·3 ml.) of the DNA solution was layered on top of 3 ml. of CsCl solution ($\rho = 1{\cdot}50$ to 1·53 in 0·01 M-Tris–HCl (pH 7·4), 0·001 M-EDTA). The tubes were covered with paraffin oil and centrifuged in a Spinco SW39L rotor at 25°C for 3 to 3·5 hr at 35,000 rev./min. Tubes were then punctured and successive fractions collected (30 to 60 fractions per tube). After addition of about 30 μg of yeast RNA as carrier to each fraction, precipitates obtained in the presence of an excess of 5% trichloroacetic acid were trapped on Whatmann GF/C filters, dried and counted in a Beckman scintillation counter.

(ii) *Alkaline CsCl gradients*

Samples of the Hirt extract, or of DNA extracted from virus, or DNA fractions recovered from other centrifugations, were centrifuged through CsCl gradients at pH 12·6 as described by Burton & Sinsheimer (1965). The collection and analysis of the fractions were as described above. Recovery of ^{3}H-label in the collected fractions generally ranged between 60 and 80% of the input radioactivity for both the neutral and alkaline CsCl runs.

(iii) *Equilibrium centrifugation in CsCl containing ethidium bromide*

Closed superhelical DNA molecules can be separated from linear or nicked circular DNA molecules by equilibrium sedimentation in CsCl containing ethidium bromide (Radloff, Bauer & Vinograd, 1967) as follows: CsCl was added (1 g/ml. of DNA solution) to the viral DNA solution (supernatant after Hirt extraction) and the mixture was centrifuged at 15,000 *g* for 20 min; the floating layer of sodium dodecyl sulfate and protein was removed with a spatula. Ethidium bromide was then added to the solution to a final concentration of 100 μg/ml. and the refractive index was adjusted to $1{\cdot}3900 \pm 0{\cdot}0010$ by adding either CsCl crystals, or buffer. The solution was then centrifuged in a fixed-angle no. 50 Spinco rotor (5 ml. per tube and filled to the cap with paraffin oil) for 48 to 60 hr at 41,000 rev./min (20°C). Fraction collection and counting were as described above. For preparative purposes, ethidium bromide was removed by successive dialysis against 2 changes of 0·01M-

Tris–HCl buffer, pH 7·4, for 6 hr, 3 changes of 0·2 M-$MgCl_2$, 2 M-urea for 48 hr and then changes of 0·1 M-Tris–HCl (pH 7·4), 0·005 M-EDTA for 24 hr. In later experiments, the dye was removed by three extractions with an equal volume of 2-propanol in the presence of CsCl, and subsequent dialysis against Tris–EDTA buffer.

(c) *Assay of DNA infectivity*

A slight modification of the method of Pagano, McCutchan & Vaheri (1967) was used for the infectivity assay. Monolayers of secondary mouse embryo cultures were infected with DNA solutions, diluted serially into Tris-buffered saline containing 100 μg of DEAE-dextran/ml. (mol. wt $\sim 2 \times 10^6$, Pharmacia Fine Chemicals, Inc., Pitcataway, N.J.). After an adsorption of 30 min, the cells were overlayed with 6·5 ml. of 0.9% agar in Eagle's medium supplemented with 3·5% horse serum. After 7 days of incubation at 31°C, a second agar-overlay (4 ml.) containing in addition 0·005% neutral red was added to the plates. The plaques were scored 18 to 22 days after the infection.

(d) *Electron microscopy of viral DNA*

DNA samples for electron microscopy were prepared by a modified spreading method of Kleinschmidt & Zahn (1959) after incubation at pH 5·2 or treatment with 10^{-3} M-hydroquinone at pH 8·5 to open supercoiled rings (Vinograd, Lebowitz, Radloff, Watson & Laipis, 1965). Electron micrographs were taken with a Siemens Elmiskop 1A, and contour lengths of the DNA molecules were measured on tracings of enlarged images with a Keuffel & Esser map measurer.

(e) *DNA–DNA hybridization*

The supercoiled polyoma DNA was first converted into a denaturable form by a limited endonucleolytic digestion (30 min incubation at 30°C with 10^{-6} μg of pancreatic DNase/ml., stabilized with 10 μg of bovine serum albumin/ml.) (Dulbecco & Vogt, 1963). Non-radioactive reference DNA (polyoma or mouse) in Tris–KCl buffer (0·01 M-Tris–HCl (pH 7·4), 0·5 M-KCl) was denatured by heating for 15 min at 100°C and quick cooling in ice. The denatured DNA solution was slowly filtered through Schleicher & Schuell B6 nitrocellulose filters (50 mm diameter), previously soaked in Tris–KCl. The filters were dried, first at room temperature, and then at 80°C in a vacuum oven for 4 hr. They were then incubated overnight in Tris–KCl buffer containing 400 μg bovine serum albumin/ml., at 60°C, and dried again at room temperature. Circular pieces (5 mm diameter) were punched out from the filters, each one containing about 0·15 μg of polyoma DNA or about 5 μg of mouse DNA; one such piece is referred to as "1 filter" in hybridization experiments. Blank filters were prepared in the same way but omitting the filtration of the denatured DNA.

Radioactive DNA, also in Tris–KCl buffer, was sonicated 4 min (setting 4 of Branson sonicator) and denatured by heating at 100°C (15 min) and quickly cooled.

DNA-containing filters and blank filters were added to glass scintillation-vials containing different amounts of radioactive DNA, in a total volume of 0·8 ml. Tris–KCl buffer (6 filters/vial). The exact input was determined by duplicate trichloroacetic acid precipitation of 100 μl. from each vial, and the vials incubated at 65°C for 20 hr. The radioactive solution was then removed and the filters washed 3 times by adding to each vial 10 ml. of 0·001 M-Tris, pH 9·0, and swirling on a Vortex Jr. mixer. After washing, the filters were dried and counted in toluene–PPO–POPOP scintillation fluid (Packard Instrument Co.). Values are the averages of 2 to 4 filters for each point.

3. Results

(a) *Kinetics of viral DNA synthesis*

(1) *Induction of viral DNA synthesis following shift from 38·5 to 31°C*

In the preceding paper, Vogt (1970) has shown that the transformed clone, Ts-a-3T3, produces very little virus ($< 10^5$ p.f.u./ml.†) when growing at 38·5°C. Following a shift

† Abbreviations used: p.f.u., plaque-forming units; M-, D- and T- forms, mono-, di- and trimers of polyoma DNA.

of the culture from 38·5°C to 31°C, virus production rises sharply after about 36 hours and increases with time until, about 100 hours after the shift, titers of about 2×10^8 p.f.u./ml. are reached. However, induction of virus multiplication does not occur at the same time in all cells in the culture; there is a steady increase in the percentage of cells producing viral capsid proteins between 40 and 72 hours following the temperature shift (Vogt, 1970).

Intracellular viral DNA synthesis in such cultures has been monitored with [^{3}H]thymidine as tracer and selective extraction of the viral DNA (Hirt, 1967) followed by velocity sedimentation analysis (see Methods)†. With labeling periods of 6 to 12 hours, ^{3}H-labeled viral DNA (as well as the polyoma DNA oligomers which are described in the next section) is not detectable during the first 24 hours after the culture is shifted to 31°C; after that, [^{3}H]thymidine appears in viral DNA (and its oligomers) and this incorporation continues at an increasing rate up to about 60 hours, whereupon the rate of [^{3}H]thymidine incorporation into viral DNA decreases slightly and then becomes nearly constant (Fig. 1)‡. Even with a labeling time of 24 hours and analyzing approximately ten times as much of the cellular contents by equilibrium centrifugation in cesium chloride containing ethidium bromide, we have not detected

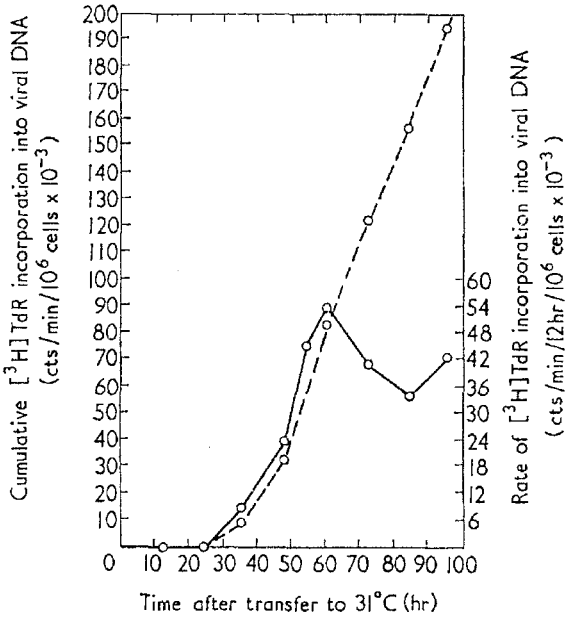

FIG. 1. Kinetics of [^{3}H]TdR incorporation into viral DNA in Ts-a-3T3 cells at 31°C.

Ts-a-3T3 cells grown at 38·5°C (5×10^6 cells/plate) were shifted at time zero to 31°C: [^{3}H]TdR was added to identical plates at successive 12-hr intervals after the shift and 12 hr later viral DNA was selectively extracted and sedimented through neutral CsCl gradients (see Methods). The amount of labeled viral DNA was estimated from the radioactivity in the two main viral bands (M and D, see Fig. 3(a)). The dotted curve shows the cumulative values (e.g., the value plotted at 48 hr is the sum of the cts/min in viral DNA found in cells labeled 0 to 12, 12 to 24, 24 to 36 and 36 to 48 hr) *versus* time after the shift. The solid line represents the rate of [^{3}H]thymidine incorporation in each pulse.

† Because in none of our experiments have sufficient quantities of viral DNA (or its oligomers) been isolated, we have no direct measure of how much of these DNA's has been synthesized at the various times after the shift to 31°C. Instead, we have relied on incorporation of [^{3}H]thymidine into DNA as a measure of DNA synthesis; we recognize, however, that the specific activity of the nucleotide pools may not be constant throughout the experimental period and, therefore, the amount of DNA synthesis during different labeling periods may not be strictly proportional to the amount of [^{3}H]thymidine incorporation.

‡ Re-infection of cells by the first virus particles produced has been minimized by having both anti-polyoma serum and receptor-destroying enzyme present in the culture throughout the incubation period at low temperature.

superhelical viral DNA before 24 hours†. Since induction of viral multiplication is not synchronous (Vogt, 1970), the constant rate of incorporation of [^{3}H]thymidine into viral DNA after 60 hours probably reflects a steady state which includes rapid synthesis in newly activated cells and declining synthesis in cells finishing their round of viral multiplication.

(2) *Synthesis of viral DNA after temperature shift*

Vogt (1970) observed that after at least eight hours at 31°C, Ts-a-3T3 cells produce a significant increase in the number of viral particles (over the background titers) when returned to 38·5°C. This can be explained in two ways:

(i) the *Ts-a* mutation blocks initiation of viral DNA replication but not the replication process itself: therefore, once the thermosensitive step has been passed in a particular cell, viral DNA can replicate in that cell at any temperature;
(ii) the *Ts-a* mutation directly effects the replication *per se* of viral DNA and, therefore, even if once initiated at low temperature, no further synthesis would occur after the temperature is raised to 38·5°C.

By the first explanation, virions recovered after a shift from 31 to 38·5°C would contain DNA synthesized at high temperature, whereas according to the second view the DNA could only have been synthesized at low temperature and subsequently encapsulated at the high temperature.

To distinguish between these two hypotheses, labeling of viral DNA at 38·5°C was measured in cells which had previously been incubated at 31°C for different periods of time. One can see (Fig. 2(a)) that cells which have been incubated at 31°C for 36 hours continue to incorporate [^{3}H]thymidine into viral DNA at high temperature at a rate similar to control cultures kept at 31°C, at least for 24 hours after the shift-up. If the cells have been at 31°C for only 24 hours, [^{3}H]thymidine labeling of viral DNA at 38·5°C continues for about 12 hours at the same rate as the control at 31°C; the rate then progressively decreases during 12 to 24 hours after the shift-up (Fig. 2(b)). Even cells which have been at low temperature for only 12 hours will synthesize labeled viral DNA during the first 12 hours after the shift-up and will continue for at least an additional 24 hours at the high temperature; control cultures, kept at 31°C for 12 hours, do not show any detectable labeling of viral DNA during the next 12 hours at 31°C.

Since the rate of [^{3}H]thymidine incorporation into cellular DNA at 38·5°C is about twice that at 31°C, one might expect a higher rate of incorporation of [^{3}H]TdR into viral DNA after shifting the activated culture to 38·5°C; however, the apparent rate of viral DNA labeling at 38·5°C is about the same as the rate found at low temperature. This could occur if only those cells which had already begun to synthesize viral DNA at the time of the shift-up continue to do so at high temperature; on the other hand, in cultures kept at 31°C, more and more cells begin to synthesize viral DNA. The

† It is conceivable that the failure to detect viral DNA is due to the insensitivity of our detection methods. Because only small quantities of viral DNA have been isolated (< 1 μg), it is difficult to evaluate accurately its specific radioactivity (cts/min/μg). However, a reasonable estimation can be made from the specific infectivity (0·3 p.f.u./ct/min) of the labeled DNA (see Table 2) and the ratio p.f.u./μg DNA (about 5×10^5 p.f.u./μg) observed in parallel experiments with larger amounts of polyoma DNA. On this basis the specific radioactivity of labeled *Ts-a* DNA is of the order of 1 ct/min/10^5 viral genomes. Assuming that we would have detected the incorporation of 1000 cts/min in the pooled contents from 10^7 cells (five small Petri plate cultures) the lower limit of detectability would be about 10 polyoma DNA molecules per cell. Even if the specific activity of the viral DNA was tenfold lower (1 ct/min/10^6 viral genomes), we might have expected to detect 100 polyoma DNA molecules per cell.

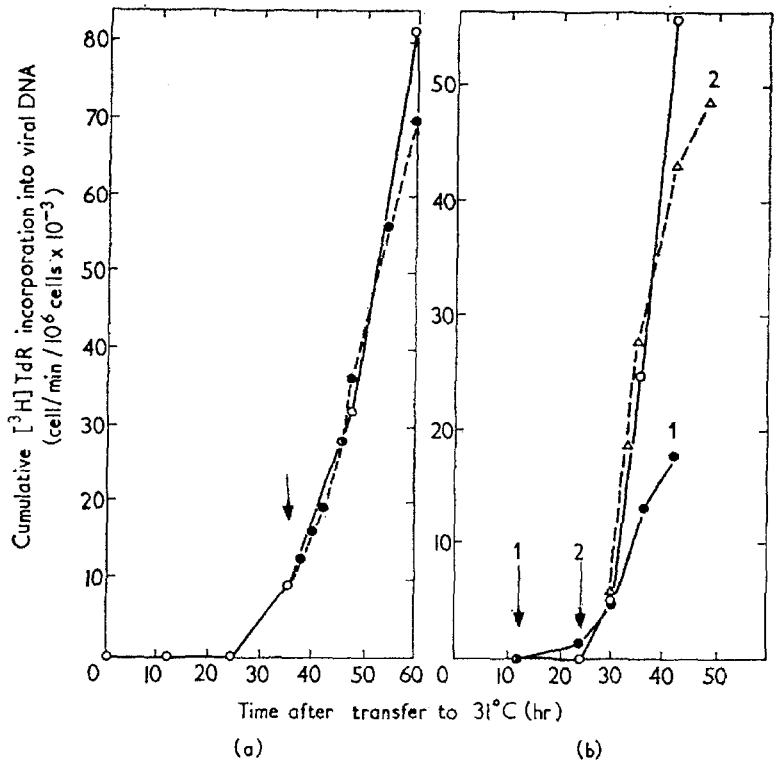

FIG. 2. Kinetics of [^{3}H]TdR incorporation into viral DNA after transfer of Ts-a-3T3 cells from 31 to 38·5°C.

The curves with open circles represent the cumulative labeling of viral DNA found after Ts-a-3T3 cells were shifted to 31°C at time zero and labeled for 6- to 12-hr periods at 31°C with [^{3}H]thymidine. At the times indicated by the arrows (36 hr in (a), 12 and 24 hr in (b)), one set of cultures was shifted back to 38·5°C and labeled for 2-, 4- or 6-hr periods; the curves with filled circles or open triangles represent the cumulative labeling of viral DNA formed at the high temperature. Viral DNA was extracted and analyzed in neutral CsCl gradients as described in the Methods section.

declining rate of viral DNA labeling at the high temperature probably reflects completion of viral DNA replication in those cells which become activated during the low-temperature incubation.

These results can be explained if the *Ts-a* function is needed in a unique event which initiates viral DNA replication in the transformed cell. Once cells have passed the critical step determined by the *Ts-a* function, they can continue to synthesize viral DNA at high temperature.

(b) *Characterization of polyoma DNA oligomers in activated Ts-a-3T3 cells*

(1) *Sedimentation properties of DNA molecules synthesized at 31°C*

(i) *Sedimentation at neutral pH.* Figure 3(a) shows the velocity sedimentation profile (in a neutral CsCl gradient) of the soluble DNA recovered by the Hirt extraction procedure from Ts-a-3T3 cells labeled with [^{3}H]TdR between 36 and 48 hours after a shift from 38·5 to 31°C: DNA from virions produced by Ts-a-3T3 cells at 31°C (Fig. 3(a)) is included as a marker. It is clear that the DNA isolated from the cell population contains mature polyoma DNA molecules; but, in addition, two faster sedimenting components not present in virions are also evident. The same pattern is obtained if the DNA fraction recovered from the cells is phenol extracted before the centrifugation. DNA recovered from Ts-a-3T3 cells kept at 38·5°C (Fig. 3(b)), shows

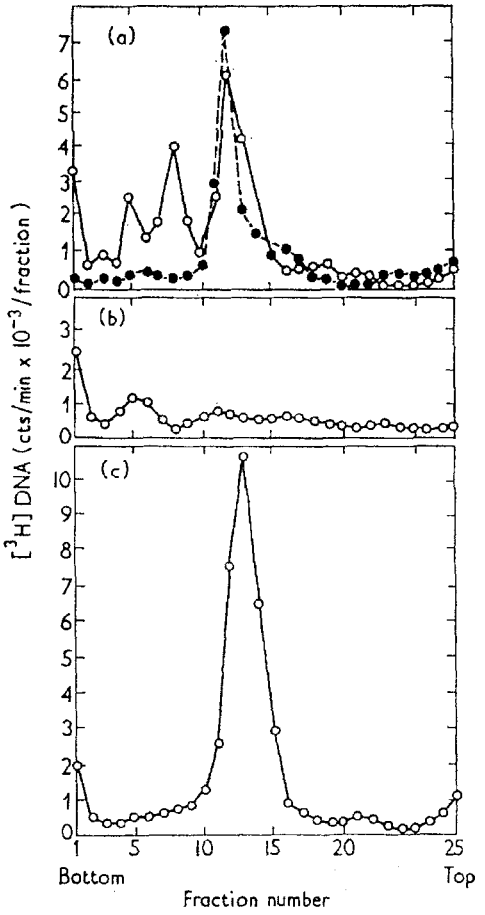

FIG. 3. Sedimentation of DNA synthesized at 31°C through neutral CsCl gradients.
(a) —○—○—, DNA extracted from Ts-a-3T3 cells 36 hr after shift to 31°C. — ● — ● —, DNA extracted with phenol from *Ts-a* virus particles made after activation of Ts-a-3T3.
(b) DNA extracted from Ts-a-3T3 cells growing at 38·5°C.
(c) DNA extracted from 3T3 cells infected with wild-type polyoma virus at 31°C.
Ts-a-3T3 cell cultures ((a) and (b)) were labeled with [^{3}H]thymidine for 12 hr, washed and the labeled DNA extracted by Hirt's procedure. Samples were sedimented through neutral CsCl gradients as described in the Methods section. In (c) the infected cells were labeled for 12 hr (48 to 60 hr after infection). The small amount of contaminating high molecular weight cellular DNA sediments to the bottom of the tube whereas T- and D-forms are found in fractions 5 and 8, respectively. Polyoma DNA form I and the M-form are in fractions 11 to 14; polyoma DNA form II is in fractions 15-17.

very much less if any of the viral type DNA molecules†; low amounts of a fast sedimenting DNA species are sometimes seen (fractions 4 to 6 (Fig. 3(b)), but they probably correspond mainly to mitochondrial DNA (Mulder & Vogt, unpublished data), the size of which is close to that of a trimer of polyoma DNA (van Bruggen, Borst, Ruttenberg, Gruber & Kroon, 1966).

The two components which sediment faster than the viral DNA are not detected in the comparable extracts of 3T3 cells infected at 37°C with wild-type polyoma (Fig. 3(c)) or with *Ts-a* virus at 31°C (the sedimentation profile is indistinguishable from that of Figure 3(c)) and labeled during the same interval. Thus, cells infected with

† See footnote on p. 321.

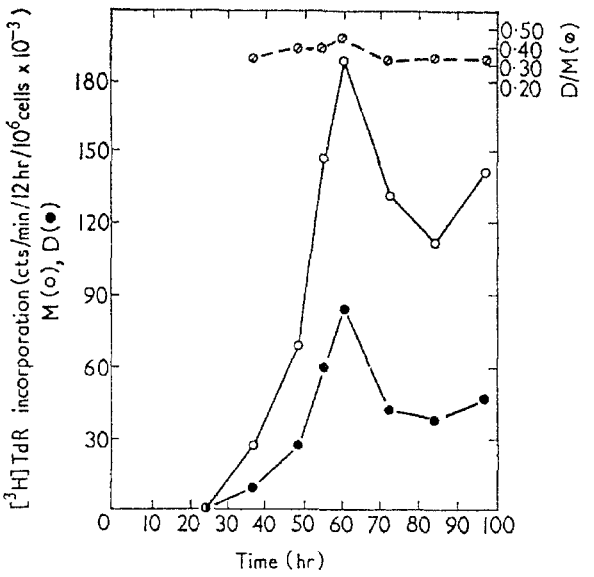

FIG. 4. Rate of [^{3}H]TdR labeling of M- and D-forms.
In an experiment identical to that reported in Fig. 1, the rates of synthesis for peaks M and D have been plotted separately.

wild-type polyoma or *Ts-a* produce almost exclusively DNA molecules of the type found as the major component in mature virions (see later).

At 31°C the relative amounts of the viral form and of the other fast sedimenting species in the activated Ts-a-3T3 cultures are constant regardless of the time of the [^{3}H]TdR labeling period after the temperature shift (Fig. 4) or the length of the labeling period (30 min to 24 hr). The three peaks in the order of the sedimentation rates comprise 3 to 10%, 25 to 30% and 60 to 70%, respectively, of the amount of labeled viral DNA.

From data to be presented in the next sections, we can identify the two faster sedimenting species of DNA as superhelical dimers and trimers of the polyoma viral DNA; we shall refer to these in the following discussions as the D-, T- and M-forms, respectively.

The relative sedimentation velocities of these three forms have been estimated from sedimentation through neutral CsCl gradients (Table 1); these measurements were made with CsCl solutions of average density $\rho = 1{\cdot}30$ instead of the usual solutions of average density $\rho = 1{\cdot}52$. It is clear that the sedimentation behavior of the faster

TABLE 1

Sedimentation behavior of the putative oligomeric polyoma DNA molecules

DNA species	Relative sedimentation rates of M-, D- and T-forms of DNA at neutral pH	Expected value†
M	1·00	—
D	1·35	× 1·38 (for dimer)
T	1·54	× 1·64 (for trimer)

† Calculated from formula $S = 7{\cdot}44 + 2{\cdot}43 \times 10^{-3} \times M^{0{\cdot}58}$ (Vinograd, personal communication).

sedimenting forms closely approximates that expected for a supercoiled molecule of two and three times the mass of the supercoiled form of polyoma DNA. The less than theoretical sedimentation velocities of the fast sedimenting species probably result from the increased buoyancy contribution of the CsCl at the bottom of the gradient.

(ii) *Sedimentation at alkaline pH.* Above pH 12·5 the sedimentation coefficient of closed circular DNA molecules increases (up to 53 s for polyoma DNA (Weil & Vinograd, 1963)), whereas linear or open circular molecules are denatured, and sediment more slowly. When extracts of Ts-a-3T3, grown and labeled with [^{3}H]TdR at 31°C, are sedimented through CsCl gradients at pH 12·6, four bands are readily discernible (Fig. 5(a)). The slowest sedimenting band corresponds to linear host DNA and nicked

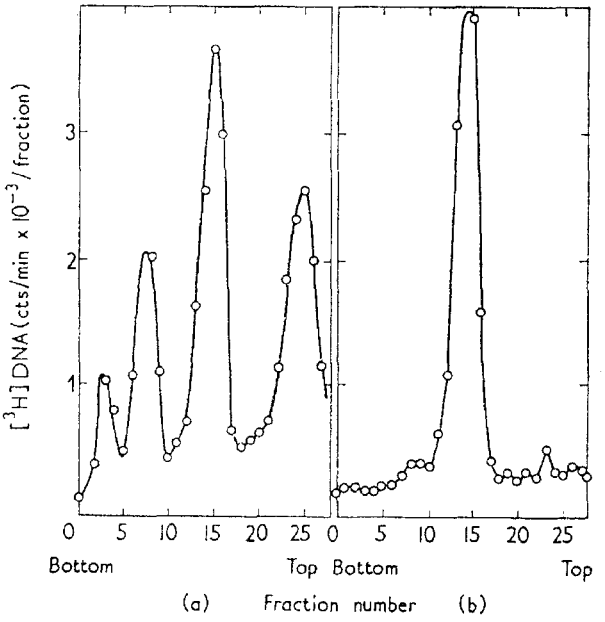

FIG. 5. Sedimentation of viral DNA extracted from Ts-a-3T3 cells through CsCl gradients at pH 12·6.

(a) The same DNA fraction used in Fig. 3(a) was centrifuged through alkaline CsCl gradients described in the Methods section. T and D peaks correspond to fractions 3 and 8, respectively. The M peak and polyoma DNA are in fractions 13 to 15; all denatured DNA molecules, derived from either linear cellular DNA or open circular molecules, sediment more slowly (fractions 23 to 28).

(b) Centrifugation under the same conditions of DNA recovered from secondary mouse embryo cultures infected at 31°C with wild-type polyoma virus and labeled during the period 20 to 70 hr after infection. This extract was first purified by banding to equilibrium in CsCl–ethidium bromide (see Fig. 6), thereby eliminating most of the denaturable DNA molecules (fractions 23 to 24).

circular DNA. The next slowest band contains polyoma viral DNA corresponding to molecules found in virions (M-form) (Fig. 5(b)). The T- and D-components isolated from a neutral CsCl gradient (see Fig. 3(a)) have sedimentation rates in alkaline CsCl corresponding to the fastest and next fastest peaks, respectively, shown in Figure 5(a). D- and T-DNA's are, therefore, very likely closed superhelical molecules. Additional support for this conclusion comes from equilibrium sedimentation in CsCl containing ethidium bromide.

(iii) *Equilibrium centrifugation in CsCl containing ethidium bromide.* Radloff *et al.* (1967) have shown that the complexes of DNA with ethidium bromide have lower buoyant densities in CsCl than does native DNA; since superhelical, covalently

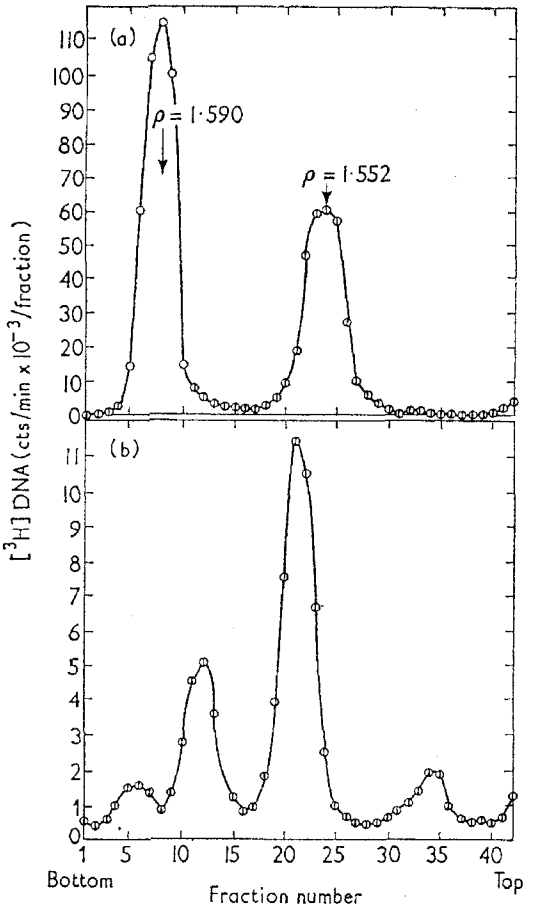

FIG. 6. Equilibrium banding in CsCl–ethidium bromide of DNA extracted from Ts-a-3T3 cells grown at 31°C.

(a) Profile of radioactivity in [^{3}H]DNA extracted from Ts-a-3T3 cells (12-hr pulse of [^{3}H]TdR 36 hr after temperature shift) in CsCl gradients containing ethidium bromide. The centrifugation and collection of fractions were as described in the Methods section.

(b) Fractions from the heavy peak shown in (a) (○) were pooled, freed of CsCl and ethidium bromide and the DNA centrifuged through a preformed alkaline CsCl gradient (same as in Fig.5(a), except that smaller fractions were collected).

closed DNA molecules bind less ethidium bromide, such complexes have a buoyant density 0·04 unit higher than do the corresponding linear or open circular molecules.

The extract prepared from Ts-a-3T3 grown and labeled with [^{3}H]TdR yields two bands at the expected densities for ethidium bromide complexes of covalently closed and linear DNA (Fig. 6(a)). The heavy band has been isolated and, after removal of the CsCl and ethidium bromide, the DNA was centrifuged through a CsCl gradient at pH 12·6 (Fig. 6(b)); the three components, M, D and T, are readily identifiable by their positions in the gradient.

(2) *Electron microscopy study of the M-, D- and T-DNA components*

Electron micrographs have been made of DNA samples extracted from Ts-a-3T3 cells grown at 31°C: material recovered from either the heavy band in CsCl containing ethidium bromide (Fig. 6(a)) or the different resolved peaks in alkaline CsCl (Fig. 6(b)) was converted into open circles (see Methods). Circular DNA of various contour

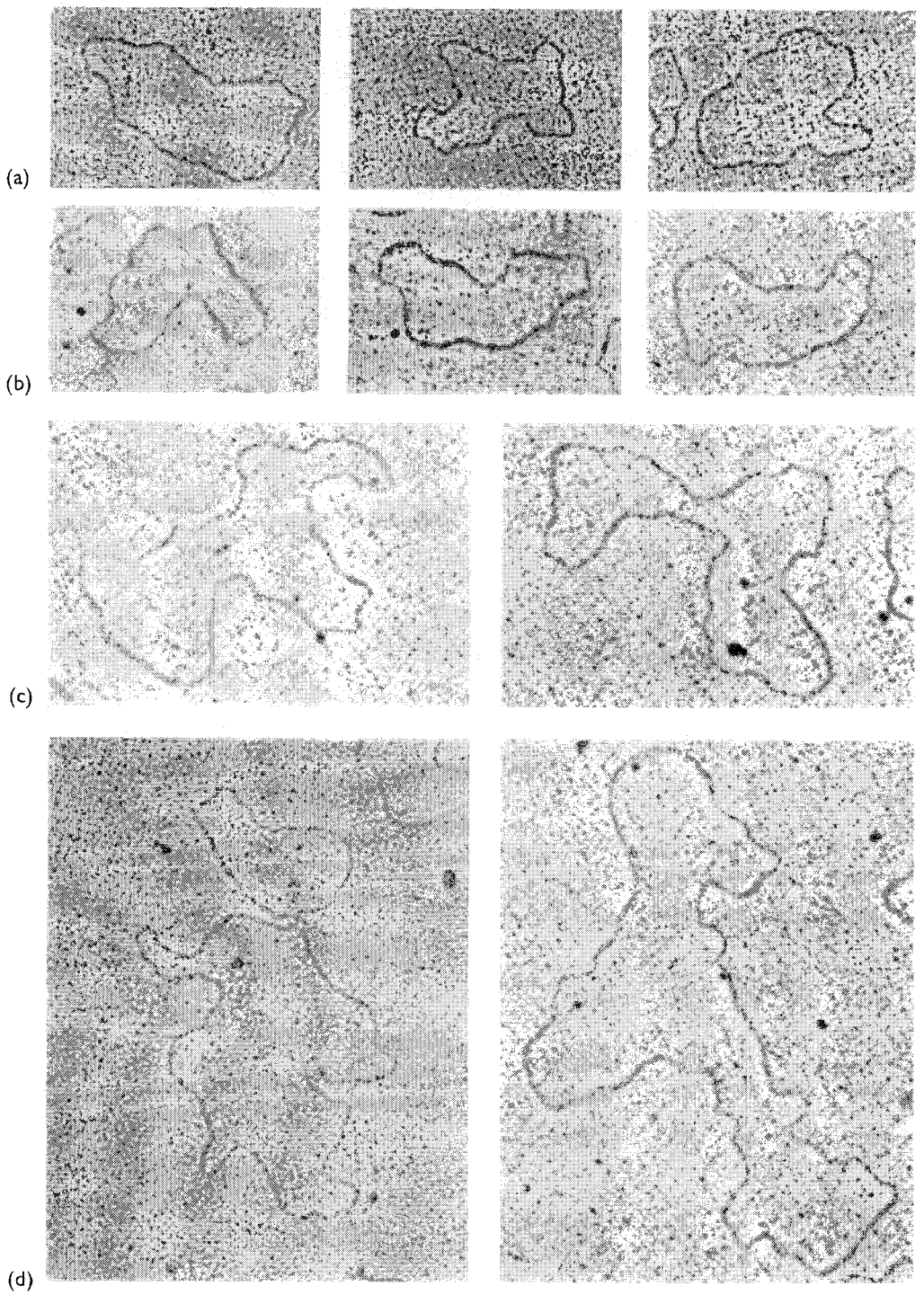

PLATE I. Electron micrographs of DNA molecules recovered from Ts-a-3T3 cells at 31°C.

Row (a) shows viral DNA extracted from cells infected with polyoma virus and purified by equilibrium sedimentation in CsCl–ethidium bromide followed by sedimentation through alkaline CsCl gradients (see Methods). The DNA was treated with hydroquinone to introduce single-strand breaks (see Methods) and renatured prior to spreading on grids.

Rows (b), (c) and (d) show the M-, D- and T-forms of polyoma DNA, respectively; these were extracted from Ts-a-3T3 cells 72 hr after transfer from 39°C to 31°C and purified as mentioned above.

[*facing page 326*

lengths is observed (Plate I). The length distributions of those molecules have been determined from enlarged pictures.

Figure 7(b) shows the distribution observed among DNA molecules extracted from activated Ts-a-3T3 cells and banded in CsCl in the presence of ethidium bromide. The mean contour lengths of the three distinct classes are in the ratios 1 : 1·92 : 2·96. The contour length distributions of the M- and D-forms which had been separated in an alkaline CsCl gradient after first sedimenting to equilibrium in CsCl–ethidium bromide are summarized in Figure 7(c) and (d).

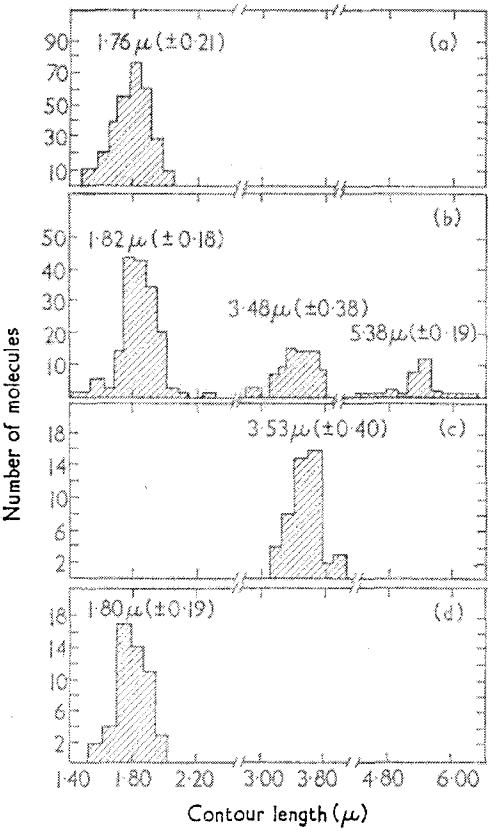

FIG. 7. Contour-length distributions in different preparations of DNA recovered from activated Ts-a-3T3. Same DNA preparations as in Plate I.

(a) Wild-type polyoma DNA;

(b) DNA extracted from activated Ts-a-3T3, after purification by equilibrium sedimentation in CsCl–ethidium bromide;

(c) and (d), same as (b), but after separation of D- and M-forms respectively, by sedimentation through alkaline CsCl gradients.

The values indicated are the mean contour lengths for each population and the numbers in parenthesis represent the standard error of that estimate.

Quite clearly, M-molecules have a contour length similar to wild-type polyoma DNA (shown in Fig. 7(a)) and D- and T-molecules are closed circles with two and three times, respectively, the size of polyoma DNA. The circular oligomers are easily distinguished from catenated forms: among several hundred electron micrographs of DNA molecules, no catenated form has been observed; moreover, unlike mitochondrial catenated DNA molecules (Hudson & Vinograd, 1967), no DNA is found at a buoyant density intermediate between that for closed and open circles after centrifugation in CsCl–ethidium bromide.

(3) *Infectivity*

The M-, D- and T-forms, after isolation by selective extraction, banding in CsCl–ethidium bromide and sedimentation through alkaline CsCl gradients, were assayed for infectivity on secondary mouse embryo cultures, as described under Methods (Table 2). The specific infectivity of each fraction is expressed as the ratio of plaque-

TABLE 2

Infectivity of isolated polyoma monomers dimers and trimers

Fraction	p.f.u./ml.	cts/min/ml.	Specific infectivity p.f.u./cts/min
Experiment 1			
M	$3{\cdot}7 \times 10^4$	$1{\cdot}1 \times 10^5$	0·34 (1·0)
D	$6{\cdot}6 \times 10^3$	$4{\cdot}2 \times 10^4$	0·16 (0·5)
T	$1{\cdot}7 \times 10^3$	$1{\cdot}4 \times 10^4$	0·12 (0·3)
Experiment 2			
M	$5{\cdot}3 \times 10^3$	$1{\cdot}7 \times 10^4$	0·31 (1.0)
D	$6{\cdot}0 \times 10^2$	$5{\cdot}8 \times 10^3$	0·10 (0.3)
T	$6{\cdot}5 \times 10$	$1{\cdot}4 \times 10^3$	0·05 (0.2)

The M-, D- and T-forms were isolated by velocity sedimentation in alkaline CsCl (see Methods and Fig. 6(b)). The infectivity and radioactivity of each DNA preparation was determined as described in the Methods section.

forming units to ^3H counts. All three forms are infectious and the specific infectivities of D- and T-forms have ranged between 0·3 to 0·5 and 0·2 to 0·3, respectively, that of the M-form (Table 2). This result shows that both the D- and T-DNA molecules contain at least one complete polyoma genome equivalent.

(4) *Hybridization*

Although D- and T-DNA molecules are infectious, and two and three times, respectively, the size of polyoma DNA, they could contain, in addition to the one genome equivalent of polyoma, additional cellular DNA. To rule out such a hypothesis, DNA–DNA hybridizations have been performed.

If D- and T-molecules contain segments of labeled mouse DNA sequences, one would expect significant hybridization of these molecules with mouse DNA. On the other hand, if they contain a specific region only, and therefore only a very small fraction of the total host DNA sequences, hybridization experiments would not be sensitive enough to detect it. But, in any case, if the oligomers contain sequences other than from polyoma, the specific hybridizability of these fractions with polyoma DNA would decrease; that is, for a given amount of radioactivity one would expect half as much hybridizable material in the D-form as with the M-form, if the D molecules contained half polyoma DNA and half of any other DNA. Assuming that the three DNA forms have the same specific radioactivity, we may compare the slopes of curves representing amounts of hybridized DNA as a function of the input of either M-, D- or T-strands (provided that molecules of all types have been first degraded into

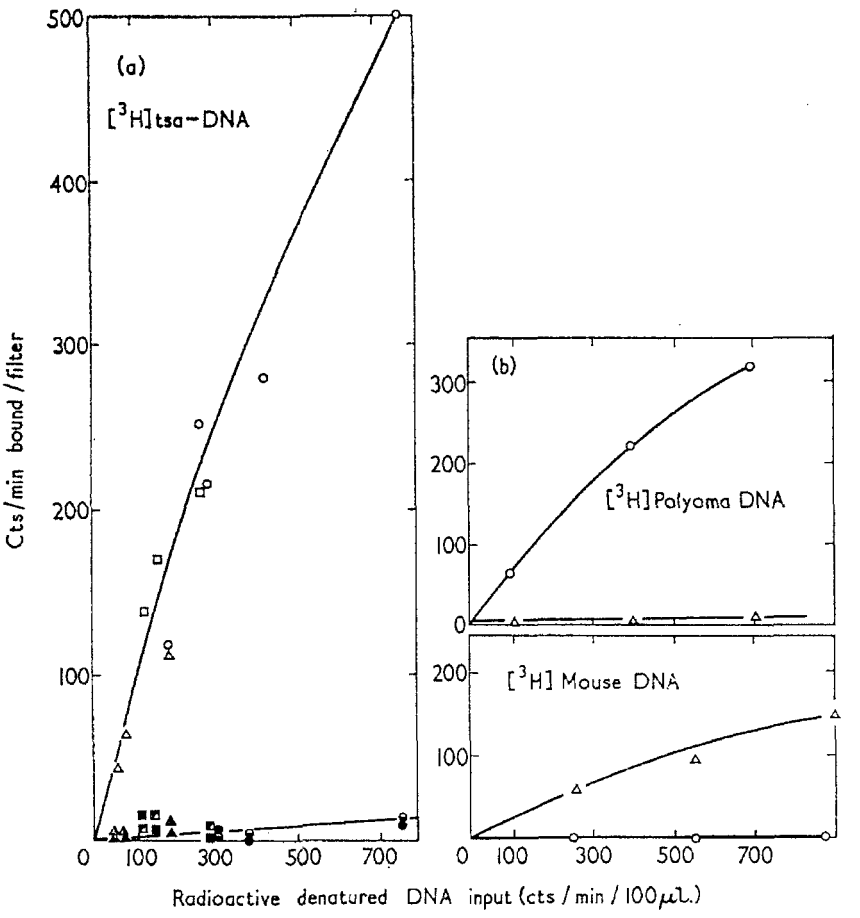

FIG. 8. Hybridization of viral DNA synthesized in Ts-a-3T3 cells with polyoma, mouse and SV40 DNA.

(a) Hybridization of radioactive monomers (○), dimers (□), and trimers (△) with polyoma DNA. Hybridization of radioactive monomers (◒), dimers (◧), and trimers (◭) with mouse DNA. Hybridization of radioactive monomers (●), dimers (■), and trimers (▲) with SV40 DNA.

(b) and (c) Controls showing hybridization of ^{3}H-labeled polyoma (b) and mouse (c) DNA's under the same conditions as in (a).

Radioactive monomers (M-form), dimers (D) and trimers (T) were isolated by Hirt's procedure from cells which had been doubly transformed with SV40 and *Ts-a* followed by equilibrium centrifugation in CsCl–ethidium bromide and velocity sedimentation at pH 12·6 (see Methods). M, D and T components used in one experiment have been all extracted from the same cell cultures.

^{3}H-labeled polyoma DNA was extracted from cells infected with wild-type virus in the presence of [^{3}H]TdR (see Fig. 6(b)), and purified by equilibrium sedimentation in CsCl–ethidium bromide. ^{3}H-labeled mouse DNA was obtained from secondary mouse embryo cell cultures labeled for 48 hr with [^{3}H]TdR (20 μc/ml.).

Unlabeled polyoma DNA was extracted from a stock of purified virus and further purified by equilibrium banding in CsCl–ethidium bromide. Unlabeled mouse DNA was recovered from confluent mouse embryo cell cultures after phenol extraction.

pieces smaller than one polyoma genome equivalent). Reconstruction experiments have shown that this method allows the detection of 10% of foreign DNA.

Hybridization was carried out with the denatured non-radioactive reference DNA (polyoma, mouse) immobilized on nitrocellulose filters, and with ^{3}H-labeled M-, D- or T-DNA in solution (Gillespie & Spiegelman, 1965; M. Green, personal communication). No cross-hybridization can be detected between mouse and polyoma DNA (Fig. 8(b) and (c)). It is clear that neither D- nor T-DNA's appreciably hybridize with mouse

DNA and that each of the forms hybridizes to the same extent with polyoma DNA over the range tested (Fig. 8(a)). These results together with the molecular size determination described above, strongly suggest that D- and T-forms are dimers and trimers, respectively, of polyoma DNA. This test, however, cannot eliminate the rather unlikely possibility that unlabeled host DNA is contained in the oligomers.

(c) *General observations on formation and utilization of oligomers*

(1) *Oligomers are not obligatory precursors of monomers, or* vice-versa

To determine if the components identified so far in Ts-a-3T3 cells at 31°C (mono-, di- and trimers of polyoma DNA) are obligatory precursors of each other, pulse–chase experiments have been performed; Ts-a-3T3 cells were first labeled with [^{3}H]thymidine for 12 hours at 31°C, then washed and transferred to a medium containing no radioactive label. Duplicate plates were harvested, either at the end of the ^{3}H pulse, or 12 hours later. The amount of label remaining in each species of polyoma DNA (monomers and oligomers) was essentially the same after the chase period. This indicates that, at 31°C, there is not a rapid inter-conversion of D- and T-forms with monomers and that these forms are not rapidly degraded. Nevertheless, since oligomers are infectious and presumably yield virions with only monomeric viral DNA molecules, it seems likely that there is a mechanism by which dimers and trimers can yield monomers.

(2) *Oligomers are not present in viral particles made after induction of virus multiplication*

In contrast to the DNA extracted from activated Ts-a-3T3 cells, the DNA extracted from virions produced in these cells shows only one component corresponding to the M-form (see Fig. 3(a)). Presumably, the dimers and trimers are excluded from virions because they contain more DNA than can be included in a polyoma capsid (Michel, Hirt & Weil, 1967).

We have also noted that after activation of Ts-a-3T3 only a small fraction (of the order of 1%) of the monomeric DNA which is synthesized finally appears in virions; the same discrepancy exists between the amount of intracellular polyoma monomer DNA and the amount subsequently appearing in progeny virus after infection with wild-type virus (our unpublished observations).

(3) *Only small amounts of oligomers are synthesized after acute infection with either wild-type or* Ts-a *polyoma.*

The main component found in 3T3 cells infected with wild-type polyoma is the monomer (see Fig. 3(c)). There are, however, preliminary indications that a small amount of dimers is also produced in this case. Sedimentation of the DNA extracted from polyoma-infected cells through alkaline CsCl gradients indicates the presence of a very small band at the expected position of viral dimers (see Fig. 5(b)). Resedimentation of this band yielded a population of infectious molecules sedimenting as dimers (Mulder & Vogt, unpublished results). Also, we have observed in electron micrographs of this material the occasional presence of closed molecules of twice the contour length of polyoma DNA. However, their frequency does not exceed, at most, 1 to 2% of the total viral DNA, and trimers, if present, are too rare to be detected. The same situation is observed after infection of 3T3 cells or secondary mouse embryo cultures, with *Ts-a* virus at 31°C. These experiments have been done using either virus stocks

derived from the original *Ts-a* isolates of Fried (1965*a*) or virus preparations recovered by activation of Ts-a-3T3 cells at low temperature.

Synthesis of large amounts of oligomers is, therefore, a specific consequence of the induction of virus multiplication in the transformed cell, Ts-a-3T3, by a temperature shift and is not a normal feature of polyoma DNA replication, or of replication of this particular mutant.

4. Discussion

Fried, in earlier experiments (1965*b*), and Eckhart more recently (1969), showed that the *Ts-a* mutant of polyoma fails to transform BHK cells morphologically at 38·5°C, but normal transformation frequencies were found at 31°C. Once transformed, however, there is neither induction of viral multiplication nor loss of the transformed phenotype when the Ts-a-BHK cells are propagated at 31 or 38·5°C. As shown by Vogt (1970), the situation is clearly different in the transformed line Ts-a-3T3. In these cells maintenance of the transformed phenotype requires that the *Ts-a* function be inoperative; if the *Ts-a* function is expressed, replication of the viral genome is induced, both as a monomeric genome and as various sized oligomers.

How can we explain the formation of relatively large quantities of polyoma oligomers following activation of Ts-a-3T3? The failure to find appreciable amounts of viral DNA oligomers after infection of 3T3 cells with *Ts-a* suggests that neither replication of Ts-a-DNA *per se*, nor recombinational processes in these cells, can account for the occurrence of circular oligomers after activation. Our speculations have considered two quite different explanations; both can account for oligomer formation in activated cells and their virtual absence in acutely infected cells. The two models differ in their assumptions of the physical state and mode of replication of the viral genome in the Ts-a-3T3 transformed cells.

The first model, the excision hypothesis, assumes that in polyoma-transformed cells the polyoma genome is covalently integrated into the host cell chromosomal DNA; and that several viral DNA copies are arranged in tandem at one or several sites in the chromosomes. Integration could occur as proposed by Campbell (1962) (see also Signer, 1968) for integration of the λ phage genome into the *Escherichia coli* chromosome. Tandem repeats would, for example, be generated if successive recombinational insertions of single polyoma chromosomes occurred within an already integrated viral sequence or if a preformed oligomer were integrated. In this model, induction of viral multiplication in Ts-a-3T3 cells would occur by excision and subsequent replication of the viral genome. Just as in the induction of λ lysogens (see Signer, 1968), excision of polyoma chromosomes could occur by the reverse of the recombinational event which led to their integration. In a structure with tandem repeats of the viral genome, "looping out" and pairing could involve variable lengths of the repeat region. With, for example, three tandem copies of the polyoma chromosome, excision could generate monomers, dimers and trimers (*via* loops of unit length 1, 2 or 3, respectively). Alternatively, different sites with different numbers of viral genomes could generate the various size oligomers. Since replication of polyoma DNA following acute infection probably does not involve integration and excision, we can understand why oligomers are generated during induction of viral multiplication in the transformed cells and only rarely during acute infection.

An alternative view of the state of the viral genome in Ts-a-3T3 growing at high temperature is that they exist as autonomous, non-integrated structures, in effect,

plasmids (of nuclear or cytoplasmic location). Conceivably, replication of this autonomous form is limited at the non-permissive temperature and occurs at a rate just sufficient to maintain several copies per cell: slow replication might result from leakiness of the *Ts-a* function or from a host mechanism. Oligomers could be formed during growth at high temperature either by mistakes in the replication process, by recombination between monomers or by other mechanisms. Thus, Ts-a-3T3, growing at 38·5°C, might already contain a population of oligomers of polyoma genomes. A shift in the temperature from 38·5 to 31°C would, therefore, initiate normal replication; viral DNA dimers and trimers, as well as monomers, would then multiply. In this model the distribution of oligomer sizes formed after activation would reflect the proportions of polyoma DNA oligomers generated during propagation of the cells at 38·5°C; the relative absence of large viral DNA molecules in acute infections is not unexpected if oligomers are formed only during growth of Ts-a-3T3 at high temperature.

Recent studies by Sambrook, Westphal, Srinivasan & Dulbecco (1968) suggest that in SV40-transformed 3T3 all of the viral DNA is covalently linked to the host DNA. No comparable information is available for the state of the polyoma DNA which is present in polyoma-transformed 3T3 (Westphal & Dulbecco, 1968). On the other hand, autonomous cirular DNA molecules are known in mitochondria (Radloff *et al.*, 1967) and in several microbial systems (F-factors (Freifelder, 1968); col E (Roth & Helinski, 1967); λ dv (Matsubara & Kaiser, 1968); P1 prophage (Ikeda & Tomizawa, 1968)). The existence of oligomers of different sizes has been reported, both for bacterial replicons (Goebel & Helinski, 1968; G. Hobom, personal communication) and for mitochondrial DNA (Clayton & Vinograd, 1969).

At present, a definite choice between these two types of models cannot be made. A critical experiment which could distinguish between them would be to determine if the viral DNA sequences in Ts-a-3T3 cells growing at 38·5°C are in the form of circular oligomers or if they are covalently linked to the host DNA. Unfortunately, spontaneous activation of *Ts-a* multiplication in a small fraction of the cells kept at 38·5°C makes such an experiment technically difficult; oligomers formed in the small number of spontaneously activated cells could not be distinguished from a small number of oligomers in all non-activated cells.

From our data we cannot assign a function to the product of the *Ts-a* gene; however, it apparently is needed early in the activation process. In the first model it might function in the recombinational event resulting in excision, and once this has been accomplished DNA synthesis proceeds even under non-permissive conditions. If we assume the same recombinational event is needed for integration (by analogy with the λ system), one can explain the failure to transform efficiently at high temperature. Since *Ts-a* DNA is only poorly replicated in acute infections at the non-permissive temperature (Eckhart, 1969; M. Fried, personal communication), we must assume that this same function is needed to initiate replication. Because polyoma viral DNA is a covalently closed, double-helical molecule, its semi-conservative replication (Hirt, 1966) requires, as does any process of integration and excision, the introduction of a break and its repair; the *Ts-a* function, if it performs either of these steps, would be required for transformation, activation and viral multiplication following infection. In the second model, induction would result from activation of any one of several possible steps needed to initiate autonomous replication.

These two models visualize that the initiating event is the reactivation of the *Ts-a*

gene product. Since a functional *Ts-a* product causes activation of the replication of the polyoma genome, we are led to inquire into the nature of other polyoma transformed 3T3 cell lines: Did they result from infections by virions with unconditional mutations in the *Ts-a* gene, or is there another reason why they are stable and virus multiplication is non-inducible?

Those of us who were visitors at the Salk Institute during this work (F. C., M. D. and P. B.) want to express our sincere appreciation to Dr Renato Dulbecco for the hospitality of his laboratory. He and his colleagues provided the help and encouragement that made our stay so enjoyable. Each of us is also grateful to Drs Dulbecco, Eckhart, Sambrook and Mulder for the many helpful discussions and criticisms during the experimental and writing phases of the work.

Some of the work described in this paper was carried out in the Department of Biochemistry, Stanford University School of Medicine, Stanford, California. The work carried out at the Salk Institute was supported by a grant to Dr Renato Dulbecco from the National Cancer Institute (CA 07592); the work done in the Department of Biochemistry, Stanford University School of Medicine, Stanford, California, was supported by a grant to one of us (P.B.) from the U.S. Public Health Service, National Institutes of Health (GM 13235-04).

Much of this work was performed while one of us (P. B.) was a National Science Foundation Senior Postdoctoral Fellow (NSF 57015) and another (F. C.) was an Eleanor Roosevelt Fellow of the International Union Against Cancer.

REFERENCES

van Bruggen, E. F. J., Borst, P., Ruttenberg, G. J. C. M., Gruber, M. & Kroon, A. M. (1966). *Biochim. biophys. Acta*, **119**, 437.
Burton, A. & Sinsheimer, R. L. (1965). *J. Mol. Biol.* **14**, 327.
Campbell, A. (1962). *Advanc. Genetics*, **11**, 101.
Clayton, D. A. & Vinograd, J. (1969). *Proc. Nat. Acad. Sci., Wash.* **62**, 1077.
Dulbecco, R. & Vogt, M. (1963). *Proc. Nat. Acad. Sci., Wash.* **50**, 236.
Eckhart, W. (1969). *Virology*, **38**, 120.
Freifelder, D. (1968). *Cold Spr. Harb. Symp. Quant. Biol.* **33**, 425.
Fried, M. (1965*a*). *Virology*, **25**, 669.
Fried, M. (1965*b*). *Proc. Nat. Acad. Sci., Wash.* **53**, 486.
Gillespie, D. & Spiegelman, S. (1965). *J. Mol. Biol.* **12**, 829.
Goebel, W. & Helinski, D. R. (1968). *Proc. Nat. Acad. Sci., Wash.* **61**, 1406.
Hirt, B. (1966). *Proc. Nat. Acad. Sci., Wash.* **55**, 997.
Hirt, B. (1967). *J. Mol. Biol.* **26**, 365.
Hudson, B. & Vinograd, J. (1967). *Nature*, **216**, 647.
Ikeda, H. & Tomizawa, J. (1968). *Cold Spr. Harb. Symp. Quant. Biol.* **33**, 791.
Kleinschmidt, A. K. & Zahn, R. K. (1959). *Z. Naturf.* **14b**, 770.
Matsubara, K. & Kaiser, A. D. (1968). *Cold Spr. Harb. Symp. Quant. Biol.* **33**, 769.
Michel, M. R., Hirt, B. & Weil, R. (1967). *Proc. Nat. Acad. Sci., Wash.* **58**, 1381.
Pagano, J. S., McCutchan, J. H. & Vaheri, A. (1967). *J. Virology*, **1**, 891.
Radloff, R., Bauer, W. & Vinograd, J. (1967). *Proc. Nat. Acad. Sci., Wash.* **57**, 1514.
Roth, T. F. & Helinski, D. R. (1967). *Proc. Nat. Acad. Sci., Wash.* **58**, 650.
Sambrook, J., Westphal, H., Srinivasan, P. R. & Dulbecco, R. (1968). *Proc. Nat. Acad. Sci., Wash.* **60**, 1288.
Signer, E. R. (1968). *Ann. Rev. Microbiol.* **22**, 451.
Todaro, G. J. & Green, H. (1963). *J. Cell Biol.* **17**, 299.
Vinograd, J., Lebowitz, J., Radloff, R., Watson, R. & Laipis, P. (1965). *Proc. Nat. Acad. Sci., Wash.* **53**, 1104.
Vogt, M. (1970). *J. Mol. Biol.* **47**, 307.
Weil, R. & Vinograd, J. (1963). *Proc. Nat. Acad. Sci., Wash.* **50**, 730.
Westphal, H. & Dulbecco, R. (1968). *Proc. Nat. Acad. Sci., Wash.* **59**, 1158.